Journal of the American Mosquito Control Association, 14(2):204–209, 1998

OVICIDAL ACTIVITY OF NEEM PRODUCTS (AZADIRACHTIN) AGAINST *CULEX TARSALIS* AND *CULEX QUINQUEFASCIATUS* (DIPTERA: CULICIDAE)[1]

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ABSTRACT. Bioactive compounds contained in the seed kernel and other parts of the neem tree (*Azadirachta indica* A. Juss) have been found to show insecticidal activities and other effects in many species of insects. These activities include antifeedancy, growth regulation, fecundity suppression, male sterility, oviposition repellency, changes in biological fitness such as loss of flying ability, immunodepression, enzyme inhibition, splitting of biological rhythms, and so forth. We investigated the ovicidal effects of various formulations of azadrirachtin (AZ) against the mosquitoes *Culex tarsalis* Coquillett and *Culex quinquefasciatus* Say. The formulations tested were wettable powder Azad® WP10, emulsifiable concentrate Azad® EC4.5, and technically pure AZ. The ovicidal activity of the test neem products was influenced by concentration of AZ, age of the egg rafts, and age of the neem preparations. Other factors such as formulation and mosquito species were also involved in the degree of ovicidal activity. When the egg rafts were deposited directly in fresh neem suspension and left there for 4 h before transfer to untreated water, 1 ppm of AZ produced almost 100% mortality in eggs. When egg rafts aged for 0, 4, 8, 12, and 24 h were exposed to 10 ppm neem suspensions for 36 h, the ovicidal activity was only attained in the egg rafts deposited directly (0 h old) in the neem suspension, not in those with ages of 4–24 h. On aging, depending on the formulations and mosquito species, the neem suspensions at 1 ppm completely lost ovicidal activity within 7–20 days. The egg rafts of *Cx. quinquefasciatus* were more susceptible to the test neem products than those of *Cx. tarsalis*. The formulated neem products were more persistent and effective than the technical AZ. The wettable powder (WP) formulation was slightly more persistent and effective than the emulsifiable concentrate (EC). The ovicidal activity of the neem products against mosquitoes from the current research clearly demonstrated the potential of neem products as possible ovicides against *Culex* mosquitoes.

KEY WORDS Neem, *Azadirachta indica*, azadirachtin, ovicide, *Culex tarsalis*, *Culex quinquefasciatus*

INTRODUCTION

Interest in the development of botanical insecticides started in the early 1930s and was sustained through the late 1950s (Campbell et al. 1933, Haller 1940, Wilcoxon et al. 1940, Hartzell and Wilcoxon 1941, Jacobson 1958). This effort was halted after that time because of the appearance, development, and use of synthetic insecticides. However, interest in botanical pesticides revived during recent years because of some of the drawbacks of synthetic insecticides, including lack of selectivity, impact on the environment, and the emergence and spread of pest resistance. At the present time, isolation, identification, and development of natural products are the focus of numerous research programs around the globe. To date, about 2,000 plant species have been reported to possess pest control properties (Ahmed et al. 1984), and of these about 344 species of plants have been studied and found to contain bioactive materials showing some activity against mosquitoes (Sukumar et al. 1991). The most prominent phytochemical pesticides found in recent years are those based on the neem tree (*Azadirachita indica* A. Juss) products, of which the dominant component possessing pesticidal properties is a steroidlike tetranotriterpenoid, azadirachtin (AZ). The properties and bioactivities of AZ and related principles have been investigated in the field of phytochemistry and entomology. It has been found that AZ and other bioactive compounds from neem extracts can induce multiple effects in a variety of insect species. These effects include antifeedancy, growth regulation, fecundity suppression, male sterility, changes in oviposition activity mostly as repellency, changes in fitness such as loss of flying ability, immunodepression, enzyme inhibition, splitting of biological rhythms, or even blocking the development of vector-borne pathogens in the arthropods. Recent advances dealing with the activity of neem products were reported in the comprehensive reviews by Schmutterer (1988, 1990), Ascher (1993), and Mordue and Blackwell (1993). A number of commercial formulations of AZ have now been developed and registered for the control of a variety of phytophagous insects.

Past experience has shown that there is little interest in developing and commercializing a product solely for use against mosquitoes. In the course of the development of mosquito larvicides and adulticides, it has always been difficult to register a product for mosquito control alone. Manufacturers and industries involved in pesticide screening, development, and commercialization first find a niche for their products in agriculture, household, or forestry uses before they encumber expenditures to promote the product for mosquito control. The pub-

[1] These studies were supported by University of California Special Funds for Mosquito Research.

lic now desire and demand natural products and biopesticides of biological origin that they perceive as safe and environmentally acceptable. The best candidates in this category of products are botanical pesticides, especially those based on neem tree products. Taking the advantage of neem research achievements in the control of agricultural pests, it is now the time to explore the potential usefulness of some of the commercial neem formulations for mosquito control.

Recent studies with laboratory crude extracts or technically pure AZ have indicated that AZ-rich fractions and related neem components were effective against mosquitoes. These products primarily act as larvicides (Attri and Prasad 1980; Chavan 1984; Zebitz 1984, 1986; Chavan and Nikam 1988; Rao et al. 1988, 1995; Naqvi et al. 1991; Sagar and Sehgal 1996; Mulla et al. 1997). Neem products have also been reported to suppress reproduction (Dhar et al. 1996, Ludlum and Sieber 1988). Additionally, neem products have been shown to exhibit repellency against adult mosquitoes, suppressing landing and biting activity of host-seeking mosquitoes (Sharma and Ansari 1994; Sharma et al. 1993a, 1993b). In addition to being a potential larvicide, it is possible that AZ may possess other properties such as ovicidal action in mosquitoes. No information is available regarding ovicidal activity of AZ and related products. The current research was carried out to investigate this activity of 2 neem formulations, wettable powder Azad™ WP10 and emulsifiable concentrate Azad™ EC4.5, as well as technically pure azadirachtin (AZ) against *Culex tarsalis* Coquillett and *Culex quinquefasciatus* Say in the laboratory.

MATERIALS AND METHODS

Mosquito colony handling

The test species were *Cx. tarsalis* and *Cx. quinquefasciatus*. Larvae were reared in 2 separate culture rooms maintained at 26 ± 1°C, 40–60% relative humidity (RH), and a 14:10 h light : dark (L:D) photoperiod with 1-h dawn and dusk periods. To rear larvae for testing, 4–5 egg rafts were placed in an enamel pan (40 × 24 × 6 cm) containing 2,500 ml distilled water. The larvae were fed dry powder food at the doses of 80, 160, 320, and 240 mg per pan every day for 1st-, 2nd-, 3rd-, and 4th-instar larvae, respectively. The larval food consisted of powdered rat chow and brewer's yeast in the ratio of 3:1. Water was added every other day to replenish loss due to evaporation. Pupae were removed from the pans and placed in screened cages (23 × 23 × 32 cm) where the adults emerged. Adults were provided continuously with 10% sucrose solution in a plastic jar provided with a cotton wick. On day 5 postemergence, the adults were deprived of sugar feeding for 12 h, then provided with a restrained 1-week-old chick overnight for blood feeding (Animal Use Protocol No. A–M 9509052-1, University of California, Riverside, CA). Five days after blood feeding, the gravids were used in the tests.

Test materials, stock presuspensions, and concentrations

The test materials were 2 experimental formulations of neem products, wettable powder Azad WP10 and emulsifiable concentrate Azad EC4.5 (both supplied by W. R. Grace Co., Columbia, MD, now acquired by Thermo-Trilogy Co., Salt Lake City, UT), and technically pure azadirachtin (Lot 65371/1, Acros Organics, Fisher Scientific, NJ). The concentrations of AZ in Azad WP10 and Azad EC4.5 were 10% and 4.5%, respectively. These test materials, Azad WP10, Azad EC4.5, and technically pure AZ, were designated as the WP, the EC, and the technical AZ, respectively, in the following text. Stock suspensions of the formulated products were prepared in distilled water at a concentration of 0.1% (w/v) or 1,000 ppm of AZ. The technical material was dissolved in acetone (1 mg/0.5 ml) obtaining a concentration of 0.2% (w/v) or 2,000 ppm. These stock preparations were employed in ovicidal tests, where the needed aliquots were added to 100 ml of distilled water in disposable paper cups prepared for oviposition. For each test, stock suspensions or solutions were freshly prepared.

Ovicidal test

All ovicidal tests and assessment of hatching rates were carried out in a holding room maintained at 28 ± 1°C, 35–45% RH, and a 14:10 h L:D photoperiod with 1-h dawn and dusk periods. The details of each testing protocol are presented below.

Effects of AZ concentrations: For ovicidal tests using different AZ concentrations, 250 gravids were placed in a screen cage (23 × 23 × 32 cm). Six disposable cups were filled with 100 ml distilled water each, of which 5 were treated with the freshly prepared neem suspensions of the WP, the EC, or the technical AZ at 0.1, 0.5, 1, 5, and 10 ppm, with one cup left as control. The treated cups and the control were placed in the mosquito cage containing the gravids 30 min before the start of the dusk period. Within 4 h, most of the egg rafts were laid. Five of the egg rafts laid during this period were selected at random from each treated and control cup. The selected egg rafts were then transferred to untreated water cups individually for hatching after counting the eggs in each raft. This procedure was modified for 5 and 10 ppm concentrations of AZ using the EC formulation. In these 2 treatments, the gravids drowned before they could lay eggs because of the lower surface tension caused by surfactants in the formulation. In this case, freshly laid (30-min-old) egg rafts in untreated cups were transferred to the 5 and 10 ppm treat-

ments with the EC and exposed for 4 h. After this treatment, 5 egg rafts were selected at random from each treatment and control and individually transferred to untreated water cups for hatching after counting the eggs in each raft. The hatching rate of eggs was assessed 120 h after oviposition. The hatching rates were figured out as the total number of hatched larvae/the total number of eggs in all 5 egg rafts examined.

Egg raft age and ovicidal activity: Preliminary tests indicated that ovicidal activity of AZ was influenced by the age of the egg rafts. To study this relationship, 250 gravids were placed in a screen cage, where 6 oviposition cups were introduced for oviposition 30 min before the start of the dusk period. Of these 6 cups, 5 were each filled with 100 ml distilled water, and one was filled with 100 ml of 10 ppm fresh suspensions of the WP or the technical AZ. The egg rafts laid in the neem-treated cup were considered as 0 h old. These egg rafts were left in the neem suspension for 36 h, then 5 were selected at random and individually transferred to untreated water cups for hatching after counting the eggs in each raft. The hatching rate was assessed 120 h after oviposition. One cup from the other 5 cups containing distilled water was used as control where 5 egg rafts were selected at random and individually transferred to distilled water cups for hatching after counting the eggs in each raft. The hatching rate was also assessed 120 h after oviposition. The egg rafts laid in the remaining 4 cups with distilled water were used in egg raft age test after 4, 8, 12, or 24 h after oviposition, respectively. Five egg rafts of each of the ages were selected at random and individually transferred to the 10 ppm fresh neem suspensions and kept there for 36 h. After treatment, the egg rafts from each treatment were individually transferred to distilled water cups for hatching assessment, which was done at 120 h after oviposition. The hatched larvae during the treatment period, if any, were added to the total number of larvae hatched from each raft. The same modified method as that in the concentration test was employed for the test of the EC at 10 ppm against the freshly laid egg rafts. Egg rafts deposited in distilled water were exposed to the neem EC treatment for 36 h within 30 min after oviposition. After treatment, 5 egg rafts were selected at random and individually transferred to untreated water cups for hatching after counting the eggs in each raft. The hatching rate was also assessed 120 h after oviposition. The same method as in the above test was applied to calculate the hatching rate of eggs.

Effects of age of neem preparation: For testing the longevity of neem preparations, the neem suspensions at minimum effective ovicidal AZ concentrations were kept in glass jars with metal lids, and stored in the holding room for different periods. The minimum effective AZ concentration for ovicidal activity was 1 ppm, as indicated by the above concentration test. The holding room was maintained at 28 ± 1°C, 35–45% RH, and the photoperiod 14:10 h L:D (40-W fluorescent and 15-W incandescent lamps) with 1-h dawn and dusk periods (15-W incandescent only). The dimensions of the holding room were 3.1 × 2.5 × 1.4 m.

In ovicidal tests using aged neem suspensions, 250 gravids were placed in a screen cage. Four disposable cups were filled with 100 ml of 1 ppm neem suspensions of various ages, and one was filled with distilled water as a control. These 5 oviposition cups were placed in the cage containing the gravids 30 min before the dusk period started. Four hours after dusk started, 5 of the egg rafts laid in each treatment and control were selected at random and individually transferred to distilled water cups for hatching after counting the eggs in each raft. The hatching was assessed 120 h after oviposition. The tests were terminated when the aged neem suspensions were no longer effective, as indicated by hatching rate of eggs. The hatching rate of the eggs was calculated as in previous tests.

RESULTS AND DISCUSSION

Effects of AZ concentrations: In *Cx. tarsalis,* eggs did not hatch when deposited directly in the neem suspensions of 1, 5, and 10 ppm of the AZ in the WP and left there for 4 h. The same held true for 5 and 10 ppm of technical AZ. Occasional hatching occurred at 1 ppm technical AZ. The EC formulation at 5 and 10 ppm AZ reduced the water surface tension, which prevented oviposition by the gravids. Therefore, the egg rafts deposited in untreated distilled water were transferred and exposed to 5 and 10 ppm EC suspension for 4 h soon after oviposition (within 30 min). Most of the eggs hatched in these cases, but the hatching rate at 10 ppm was significantly lower than those in 5 ppm and the control. The hatching of the eggs deposited in 1 ppm of the EC was significantly lower, as compared with that in 0.1 and 0.5 ppm as well as in the control. The concentrations of 0.1 and 0.5 ppm of all test materials did not reduce egg hatch. The WP was slightly more active than the EC and the technical AZ (Table 1).

In *Cx. quinquefasciatus,* the situation was quite similar to that in *Cx. tarsalis.* The complete ovicidal activity was attained at 1, 5, and 10 ppm of the WP, 1 ppm of the EC, and 5 and 10 ppm of the technical AZ. Occasional hatching occurred at 1 ppm of the technical AZ. Some of the eggs hatched when deposited in distilled water, and soon after oviposition exposed to 5 and 10 ppm of the EC for 4 h. However, the hatching rate was significantly lower at 10 ppm than at 5 ppm and in the control. No ovicidal effect was observed at 0.1 and 0.5 ppm of all the test materials. The egg rafts of *Cx. quinquefasciatus* were slightly more susceptible to the EC than those of *Cx. tarsalis* (Table 1).

From the above results, it is quite clear that 1 ppm of AZ using all 3 neem products is the mini-

Table 1. Ovicidal activity of neem products at various azadirachtin concentrations against *Culex tarsalis* and *Culex quinquefasciatus* as indicated by the hatching rates of eggs.[1]

Formulation	Mean hatch (% ± SE) at azadirachtin concentrations (ppm)[2]					
	0.1	0.5	1	5	10	Control
			Culex tarsalis			
Azad® WP10	91.2 ± 1.36 a	78.2 ± 1.82 b	0 c	0 c	0 c	81.0 ± 1.60 b
Azad® EC4.5	78.5 ± 2.23 a	84.5 ± 2.15 a	10.7 ± 0.25 b	80.4 ± 2.19 a	46.7 ± 2.77 c	81.8 ± 2.18 a
Azadirachtin tech.	84.3 ± 2.28 a	81.7 ± 2.00 a	2.9 ± 0.20 b	0 b	0 b	86.0 ± 2.00 a
			Culex quinquefasciatus			
Azad WP10	88.0 ± 1.43 ab	82.3 ± 1.82 a	0 c	0 c	0 c	91.7 ± 1.73 b
Azad EC4.5	95.6 ± 1.17 a	82.3 ± 2.17 b	0 c	75.9 ± 2.47 b	30.0 ± 3.25 d	88.7 ± 1.85 ab
Azadirachtin tech.	85.4 ± 1.92 a	88.2 ± 2.09 a	1.1 ± 0.24 b	0 b	0 b	90.1 ± 1.88 a

[1] WP, wettable powder; EC, emulsifiable concentrate; tech., technically pure.
[2] Different letters in horizontal line indicate significant differences in hatching rate of eggs by chi-square test at 0.05 level.

mum ovicidal concentration yielding 90–100% egg mortality in fresh eggs laid in the suspensions. This concentration was almost equal in activity against both species of test mosquitoes.

Effects of egg raft age: Egg rafts with ages of 0, 4, 8, 12, and 24 h were exposed to 10 ppm of formulated neem and the technical AZ for 36 h. In all of the test materials, no ovicidal activity was noted in the egg rafts aged more than 4 h, except that occasionally partial ovicidal activity was noted in the 4- and 8-h-old egg rafts of *Cx. quinquefasciatus* in the EC formulation. In most cases, the egg rafts aged for 4 h or longer were no longer susceptible to neem products. Complete ovicidal activity was only shown in egg rafts deposited directly in the neem suspensions, and this age of the rafts is considered as 0 h here. This fact indicates that the target period of action by AZ as an ovicide is the very early stage of egg development. For the EC formulation against freshly laid eggs in both species, the egg rafts were deposited in distilled water and then transferred and exposed to neem suspension within 30 min after oviposition for assessment of ovicidal activity. In these cases, some hatching occurred that was significantly lower than that in the control. The egg rafts of *Cx. quinquefasciatus* were more susceptible to the EC than were those of *Cx. tarsalis* (Table 2). The development for even as short a period as 30 min under neem-free conditions significantly reduced the susceptibility of the egg rafts to AZ. According to these results, it is quite possible that under natural conditions, the existing neem treatment in the aquatic habitats will exert desired ovicidal activity against newly laid eggs. Conversely eggs already deposited in the aquatic habitat before treatment will not be affected ovicidally by the treatment with neem.

Longevity of neem preparations: An age-dependent decrease in ovicidal activity was discovered in all of the formulations. In *Cx. tarsalis,* the ovicidal activity of the WP decreased on day 7, and it lost its activity completely on day 15 after preparation. The activity of the EC decreased on day 1 and was lost completely on day 10 after preparation. The activity of the technical AZ decreased on day 4 and disappeared completely on day 7 after preparation. In *Cx. quinquefasciatus,* the activity of both the WP and the EC decreased on day 10 and was lost completely on day 20. The activity of the technical AZ decreased from day 4 and was lost completely on day 15 after preparation. In the neem suspensions with same ages, the hatching of the eggs of *Cx. quinquefasciatus* was overall lower than that of *Cx. tarsalis.* Therefore, it is obvious that the egg rafts of *Cx. quinquefasciatus* were more susceptible than those of *Cx. tarsalis* to the aged suspensions. The formulated neem products had a longer persistence and were more effective than the technical AZ. The WP formulation was slightly more persistent and effective than the EC formulation (Table 3). However, the longevity of the formulations under the

Table 2. Activity of neem products against *Culex tarsalis* and *Culex quinquefasciatus* eggs of various ages at the concentration of 10 ppm and exposure of 36 h as indicated by the hatching rates of egg.[1]

Formulations	Mean hatch (% ± SE) of egg rafts of ages (h)[2]					
	0	4	8	12	24	Control
			Culex tarsalis			
Azad™ WP10	0 a	78.1 ± 1.80 b	77.0 ± 2.11 b	89.3 ± 1.82 b	81.3 ± 1.81 b	84.9 ± 1.80 b
Azad™ EC4.5	21.2 ± 2.70 a	78.3 ± 2.41 b	80.2 ± 2.04 b	86.2 ± 2.19 b	86.7 ± 2.20 b	85.7 ± 2.24 b
Azadirachtin tech.	0 a	90.2 ± 1.76 b	83.5 ± 2.07 b	87.0 ± 1.95 b	90.2 ± 2.08 b	86.6 ± 1.98 b
			Culex quinquefasciatus			
Azad WP10	0 a	79.3 ± 1.77 b	84.4 ± 1.81 bc	90.4 ± 1.70 c	89.6 ± 1.39 c	82.9 ± 1.98 bc
Azad EC4.5	8.6 ± 0.40 a	79.9 ± 2.43 b	83.7 ± 2.44 b	91.2 ± 1.79 c	91.4 ± 1.68 c	92.0 ± 2.48 c
Azadirachtin tech.	0 a	85.3 ± 2.16 b	84.5 ± 2.23 b	84.9 ± 2.96 b	87.1 ± 2.47 b	84.4 ± 2.32 b

[1] WP, wettable powder; EC, emulsifiable concentrate; tech., technically pure.
[2] Different letters in horizontal line indicate significant differences in hatching rate of eggs of different age by chi-square test at 0.05 level.

Table 3. Longevity of neem product as an ovicide against *Culex tarsalis* and *Culex quinquefasciatus* at 1 ppm and treatment of 4 h from oviposition as indicated by the hatching rate of eggs.[1]

Formulation	Mean hatch (% ± SE) of eggs in neem suspensions of various ages (days)[2]							
	0	1	4	7	10	15	20	Control
				Culex tarsalis				
Azad™ WP10	1.2 ± 0.25 a	1.6 ± 0.34 a	2.4 ± 0.27 a	27.5 ± 2.31 b	76.0 ± 2.12 c	81.9 ± 1.62 cd	—	85.8 ± 2.06 d
Azad™ EC4.5	3.9 ± 0.25 a	11.6 ± 2.77 b	15.1 ± 2.24 b	31.6 ± 2.62 c	81.3 ± 2.63 d	—	—	85.6 ± 1.74 d
Azadirachtin tech.	4.4 ± 0.24 a	4.3 ± 0.24 a	59.1 ± 2.41 b	82.6 ± 1.93 c	—	—	—	83.5 ± 1.64 c
				Culex quinquefasciatus				
Azad WP10	0 a	0.7 ± 0.22 a	1.5 ± 0.27 a	6.4 ± 0.35 a	32.7 ± 2.89 b	41.3 ± 2.98 b	79.7 ± 1.97 c	82.1 ± 1.76 c
Azad EC4.5	0 a	2.4 ± 0.21 a	7.4 ± 0.21 a	7.8 ± 0.23 a	41.7 ± 2.85 b	52.7 ± 2.87 c	78.2 ± 2.06 d	86.6 ± 1.61 d
Azadirachtin tech.	—	0.9 ± 0.19 a	8.8 ± 0.28 b	24.8 ± 3.00 c	52.7 ± 2.77 d	78.7 ± 2.18 e	—	81.6 ± 1.64 e

[1] WP, wettable powder; EC, emulsifiable concentrate; tech., technically pure.
[2] Different letters in horizontal line indicate significant differences in hatching rate of eggs by chi-square test at 0.05 level.

field conditions will be shorter than that in the laboratory because of a variety of biotic and abiotic factors. An additional reason for this difference in longevity is the method for storing the neem preparation. The neem suspensions were kept in a glass jar with a metal lid, which eliminated the effects of evaporation of the neem principles, and created different effects than would be found in field situations.

The ovicidal activity of neem products against *Culex* egg rafts depended upon 3 key factors: concentrations of AZ, age of the egg rafts, and age of the neem preparations. Other factors such as formulations and mosquito species were also involved in the manifestation of ovicidal activity. In terms of concentration effects, 1 ppm AZ in fresh preparation was the minimum effective concentration for ovicidal activity in most cases. Ovicidal activity was only attained in egg rafts deposited directly in the neem suspension. The ovicidal activity of neem products against mosquitoes has practical importance, especially for *Cx. tarsalis,* where the WP acted as an oviposition attractant (Su and Mulla, submitted). The results from the current research clearly demonstrate the potential of neem products as possible ovicides against *Culex* mosquitoes, which will be an added benefit to larvicidal activity of neem products. Further investigations are needed to elucidate this activity against a wide range of mosquito species and the ovicidal mechanism involved.

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